Use of an Anti-Vascular Endothelial Growth Factor Antibody in a Pharmacokinetic Strategy to Increase the Efficacy of Intraperitoneal Chemotherapy

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ABSTRACT

The efficacy of intraperitoneal chemotherapy for ovarian cancers is limited by poor penetration of drug into peritoneal tumors. Based on pharmacokinetic theory that suggests that penetration depth is primarily determined by the rate of drug removal via tumor capillaries, we have hypothesized that co-administration of antiangiogenic therapy will allow for decreased drug removal, increased drug concentrations in tumor, and increased efficacy of intraperitoneal chemotherapy. Pharmacokinetic modeling was conducted to simulate the effect of tumor blood flow on tumor concentrations of topotecan. Simulations predicted that tumor blood flow reductions, as potentially achieved by antiangiogenic therapy, would lead to substantial increases in tumor concentrations after intraperitoneal chemotherapy but would lead to a slight decrease after systemic chemotherapy. Pharmacokinetic studies performed using the A2780 xenograft tumor model showed that animals receiving combined intraperitoneal topotecan and an anti-vas-

cular endothelial growth factor (VEGF) monoclonal antibody had ~6.5-fold higher ($p = 0.0015$) tumor topotecan concentrations compared with animals receiving intraperitoneal topotecan alone, whereas there was no significant ($p = 0.16$) difference for systemic topotecan. Therapeutic studies conducted with two different drugs, topotecan and cisplatin, showed that animals receiving combined intraperitoneal chemotherapy and anti-VEGF therapy displayed superior survival relative to animals treated with chemotherapy alone (i.e., cisplatin or topotecan), anti-VEGF alone, or intravenous chemotherapy with concomitant anti-VEGF therapy. Combined intraperitoneal topotecan and anti-VEGF resulted in the complete cure of four of 11 mice. The proposed combination of antiangiogenic therapy and intraperitoneal chemotherapy, which was predicted to be beneficial by pharmacokinetic simulations, may provide substantial benefit to patients with peritoneal malignancies.

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Intraperitoneal chemotherapy has been demonstrated to achieve peritoneal drug concentrations that are typically 1 to 3 logs higher than concentrations attained with systemic drug administration (Dedrick et al., 1978). Despite the high local concentration of drug, intraperitoneal chemotherapy often fails to produce complete and lasting responses in the treatment of experimental and human peritoneal tumors (Kawai et al., 1994; Sautner et al., 1994; Rosenthal and Jacobs, 1998). One of the main factors limiting the clinical success of intraperitoneal chemotherapy is the modest penetration of drug into tumor tissue (Ozols et al., 1979; McVie et al., 1982; Los et al., 1989; Dedrick and Flessner, 1997; Waniewski et al., 1999). For example, Ozols et al. (1979) used fluorescence microscopy after intraperitoneal doxorubicin administration in tumor-bearing mice to demonstrate that measurable drug concentra-

ABBREVIATIONS: VEGF, vascular endothelial growth factor; PBPK, physiologically based pharmacokinetic; HPLC, high-performance liquid chromatography; PBS/T, 0.05% Tween 20 in phosphate-buffered saline; HPF, high-power microscopic field; CI, confidence interval; K_{in} , steady-state rate of drug entry into the tumor; CL, clearance; Q, tumor blood flow.

tions were present only in the outermost four to six cell layers in solid intra-abdominal tumors. Likewise, Los et al. (1989) found that tumor concentrations of cisplatin were significantly elevated 1 mm from the peritoneal surface, but not at 1.5 mm, after intraperitoneal cisplatin administration in a rat model.

The Distributed Model was introduced by Dedrick and Flessner (Flessner et al., 1984) to characterize and predict drug penetration in tissues after intraperitoneal chemotherapy. For the sake of simplicity, the model assumes that blood capillaries are uniformly distributed within tissues at various distances from the peritoneal surface (Fig. 1). The model predicts that the penetration of drug into tissue is limited by removal of drug by capillaries perfusing the tissue. Gupta et al. (1995) extended this work by relating a first-order elimination rate constant (k), which describes the elimination of drug from the tumor by diffusion into blood capillaries, to its mechanistic determinants (i.e., drug permeability for capillaries p ; the surface area of capillaries in tissue, a ; and blood flow through the tissue, q):

$$k = \frac{p \cdot a \cdot q}{p \cdot a + q} \quad (1)$$

Based on this relationship, it follows that any reduction in p , a , or q will decrease the value of k and, consequently, increase the penetration of drug into tumor tissue.

Antiangiogenic therapy has been shown to decrease tumor vessel diameter, density, and permeability (Jain, 2001; Hu et al., 2002). Hence, we hypothesized that the use of antiangiogenic therapy in combination with intraperitoneal chemotherapy will increase the depth of drug penetration into peritoneal tumors. Given that the effect of antiangiogenic agents is limited to developing blood vessels, we hypothesized that the increased depth of penetration would be observed in developing tumors but not in normal organs with established vasculature. In addition, antiangiogenic treatment may be expected to decrease the average size of tumors (Folkman, 1990), which should also

translate to improved drug exposure in tumors after regional chemotherapy (i.e., by decreasing the distance required for drug distribution throughout the tumor).

Work presented in this article includes computer simulations that predict the effect of changes in tumor blood flow on the tumor penetration of a chemotherapeutic drug, topotecan. Also presented are a series of in vivo investigations that assess the effect of anti-vascular endothelial growth factor (VEGF) on tumor burden, tumor blood vessel density, tumor penetration of topotecan, and on the efficacy of topotecan and cisplatin chemotherapy in a xenograft model of human ovarian cancer.

Materials and Methods

Materials. Cisplatin and the anti-VEGF monoclonal antibody (Bevacizumab; Genentech, South San Francisco, CA) were purchased from a local pharmacy. Triethylamine, acridine, potassium dihydrogenphosphate, phosphoric acid, and methanol were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade. ALZET micro-osmotic pumps 1003D were obtained from Alza (Palo Alto, CA). A2780 cells were obtained from the DTP, DCTD Tumor Repository (maintained by Charles River Laboratories, Inc., under contract to the Biological Testing Branch for the National Cancer Institute, Frederick, MD). Cells were cultured in an incubator with 5% CO₂ at 37°C using RPMI 1640 medium (20 mM HEPES, 2 mM L-glutamine) mixed with 10% heat-inactivated fetal bovine serum and 5 mg/l gentamicin. Protocols for animal use were approved by the Institutional Animal Care and Use Committee of the University at Buffalo. Mice were housed in a sterile room on a standard light/dark cycle, with continuous access to food and water.

Pharmacokinetic Model for Simulation. Topotecan disposition was simulated with the use of a hybrid physiologically based pharmacokinetic (PBPK) model (Fig. 2). Simulations evaluated the effect of changes in peritoneal tumor blood flow on tumor penetration of topotecan after intravenous or intraperitoneal administration. The model includes a peritoneal compartment, two systemic disposition compartments, and a physiologically based tumor model. As with our previous modeling of topotecan disposition in mice (Chen et al., 2007), the model predicts that a fraction of topotecan in the peritoneal bulk fluid will undergo linear presystemic elimination, which is described by a first-order rate constant (k_{el}). Topotecan may bypass elimination and gain entry into the systemic circulation (i.e., the central compartment) as described by the distribution clearance and the peritoneal bioavailability [CL_{DP}/F]. Drug in the central compartment is available for distribution back into the peritoneal compartment, distribution into the peripheral compartment, or for elimination as described with a nonlinear, capacity-limited function (e.g., consistent with saturable process such as active renal secretion). Drug in the peritoneal compartment may penetrate into peritoneal tumors, which has been represented with the physiologically based “sphere model.” The tumor model assumes a spherical tumor with a diameter of 1 cm. The tumor is divided into five hypothetical layers, A to E, with equal thickness (1 mm). Each tumor layer is linked to the central compartment through blood flow, which is described as Q_i , for the “ i th” layer. Moreover, each tumor layer is connected to adjacent layers using a permeability surface area coefficient (PS_{ij} , where “ ij ” refers to two adjacent tumor layers). The outer most tumor layer, layer A, is connected to the peritoneal compartment via PS , which is described as PS_{PA} . Differential eqs. 2 through 9 describe the hybrid PBPK model:

$$\begin{aligned} \frac{dC_1}{dt} = & \frac{CL_D}{V_1} \times (C_2 - C_1) - \frac{V_{max}}{K_m + C_1} \times \frac{C_1}{V_1} + \frac{CL_{DP}}{F \times V_1} \times (C_p - C_1) \\ & + \frac{Q_A}{V_1} \times (C_{TA} - C_1) + \frac{Q_B}{V_1} \times (C_{TB} - C_1) + \frac{Q_C}{V_1} \times (C_{TC} - C_1) + \frac{Q_D}{V_1} \\ & \times (C_{TD} - C_1) + \frac{Q_E}{V_1} \times (C_{TE} - C_1) \quad (2) \end{aligned}$$

Fig. 1. The distributed model. The model assumes that blood capillaries (hollow circles in the figure) are uniformly distributed within tissues at various distances from the peritoneal surface, and the model predicts an exponential decrease in drug concentration with increasing distance from the peritoneal surface. C_p , concentration of unbound drug in the peritoneal fluid; C_t , unbound drug concentration in the tumor. C_t declines exponentially with distance from the tumor surface, as shown by the solid line. X_0 is the depth at which the concentration difference between drug at the surface of the tumor and drug in the tumor has decreased to 37% of its maximal value. $3X_0$, distance from the surface of the tumor where drug concentration difference is 5% of its maximal value. Adapted from Dedrick and Flessner (1997).

Fig. 2. The pharmacokinetic model for simulations. The model includes a peritoneal compartment, two systemic disposition compartments, and a physiologically based tumor model. Drug in the peritoneal compartment may penetrate into peritoneal tumors, which have been represented with the physiologically based sphere model. The tumor model assumes a spherical tumor with a diameter of 1 cm. The tumor is divided into five hypothetical layers, A to E, with equal thickness (1 mm). Each tumor layer is linked to the central compartment through blood flow, which is described as Q_i , for the i th layer. Each tumor layer is also connected to adjacent layers using a permeability surface area coefficient (PS_{ij} , where ij refers to two adjacent tumor layers). The outer most tumor layer, layer A, is connected to the peritoneal compartment via PS_{PA} . C_1 , C_2 , and C_P represent concentrations of topotecan in the central, peripheral, and peritoneal compartments. C_{TA} , C_{TB} , C_{TC} , C_{TD} , and C_{TE} represent topotecan concentrations in tumor layers A to E, respectively. V_1 , V_2 , and V_P represent the apparent volumes of distribution for the central, peripheral, and peritoneal compartments. The physical volumes of tumor layers are described as V_A , V_B , V_C , V_D , and V_E .

$$\frac{dC_2}{dt} = \frac{CL_D}{V_2} \times (C_1 - C_2) \quad (3)$$

$$\frac{dC_P}{dt} = \frac{K_0 \times F}{V_P} - \frac{CL_{DP}}{V_P} \times (C_P - C_1) - k_{el} \times C_P - \frac{PS_{PA} \times F}{V_P} \times (C_P - C_{TA}) \quad (4)$$

$$\frac{dC_{TA}}{dt} = \frac{Q_A}{V_A} \times (C_1 - C_{TA}) + \frac{PS_{PA}}{V_A} \times (C_P - C_{TA}) - \frac{PS_{AB}}{V_A} \times (C_{TA} - C_{TB}) \quad (5)$$

$$\frac{dC_{TB}}{dt} = \frac{Q_B}{V_B} \times (C_1 - C_{TB}) + \frac{PS_{AB}}{V_B} \times (C_{TA} - C_{TB}) - \frac{PS_{BC}}{V_B} \times (C_{TB} - C_{TC}) \quad (6)$$

$$\frac{dC_{TC}}{dt} = \frac{Q_C}{V_C} \times (C_1 - C_{TC}) + \frac{PS_{BC}}{V_C} \times (C_{TB} - C_{TC}) - \frac{PS_{CD}}{V_C} \times (C_{TC} - C_{TD}) \quad (7)$$

$$\frac{dC_{TD}}{dt} = \frac{Q_D}{V_D} \times (C_1 - C_{TD}) + \frac{PS_{CD}}{V_D} \times (C_{TC} - C_{TD}) - \frac{PS_{DE}}{V_D} \times (C_{TD} - C_{TE}) \quad (8)$$

$$\frac{dC_{TE}}{dt} = \frac{Q_E}{V_E} \times (C_1 - C_{TE}) + \frac{PS_{DE}}{V_E} \times (C_{TD} - C_{TE}) \quad (9)$$

C_1 , C_2 , and C_P represent concentrations of topotecan in the central, peripheral, and peritoneal compartments. In addition, C_{TA} , C_{TB} , C_{TC} , C_{TD} , and C_{TE} represent topotecan concentrations in tumor layers A, B, C, D, and E, respectively. V_1 , V_2 , and V_P represent the apparent volumes of distribution for the central, peripheral, and peritoneal compartments. The physical volumes of tumor layers are

described as V_A , V_B , V_C , V_D , and V_E . In eq. 2, V_{max} is the maximal rate of topotecan elimination, and K_m is a Michaelis-Menten constant. K_0 in eq. 4 represents a zero-order infusion rate constant for topotecan administration into the peritoneal cavity.

Parameter values used for the simulation with the hybrid PBPK model are described in Table 1 (Chen et al., 2007). Permeability surface area coefficients were calculated from the equation: $PS = D \times A/W$, where D is the tissue diffusivity of topotecan, A is the surface area of the tumor layer, and W is the thickness (1 mm) of the tumor layer. Tissue diffusivity of topotecan was calculated based on its molecular mass (421 Da) using the equation $D = 0.366 \times \text{molecular mass}^{-0.45} \times 10^{-5} \text{ cm}^2/\text{s}$ (Waniewski et al., 1999), and it was found to be $2.42 \times 10^{-7} \text{ cm}^2/\text{s}$. The volume and surface area for each tumor layer were calculated based on equations for a sphere. The value of blood flow to the tumor, 0.06 ml/min/g, was obtained from the literature (Waniewski et al., 1999) and was individualized for each tumor layer based on the volume of the layer. The density of the tumor is assumed to be 1 g/ml, and it was assumed that there was no specific binding of topotecan in tumor tissue. All simulations were based on an animal weight of 25 g. Topotecan was infused either intraperitoneally or intravenously at the dose of 15 mg/kg over the period of 72 h, which is the maximum tolerated dose for 72-h infusion (J. Chen, D. K. Shah, and J. P. Balth, unpublished data). To simulate the effect of antiangiogenic therapy on tumor blood perfusion, different simulations were performed with varying values of tumor blood flow. It is reported that antiangiogenic therapy can reduce blood vessel density by up to 75% (Liang et al., 2006), and, for the purpose of simulation, the normal tumor blood flow value of 0.06 ml/min/g was reduced by 50, 75, and 90%. All simulations were performed with ADAPT-II software (University of Southern California, Biomedical Simulations Resource, Los Angeles, CA).

Pharmacokinetic Experiments to Study Tumor Drug Exposure. Two separate animal studies, studies A and B, were performed to examine the effect of anti-VEGF (United States Food and Drug

TABLE 1

Parameter values used for simulations using the hybrid physiologically based pharmacokinetic model (for more details, see text)

Parameter (Unit)	Value	Parameter (Unit)	Value
V_1^* (ml)	80.8	PS_{PA}	0.0273
V_2^* (ml)	1.00E+03	PS_{AB}	0.0175
V_{max}^* (ng/h)	9.58E+04	PS_{BC}	0.0098
K_m^* (ng/ml)	1780	PS_{CD}	0.0044
CL_D^* (ml/h)	55.8	PS_{DE}	0.0011
k_{el}^* (1/h)	4.22	Q_A	0.920
CL_{DP}/F (ml/h)	0.561	Q_B	0.558
V_P/F (ml)	0.321	Q_C	0.287
V_A (ml)	0.256	Q_D	0.106
V_B (ml)	0.155	Q_E	0.015
V_C (ml)	0.080		
V_D (ml)	0.029		
V_E (ml)	0.004		

* Pharmacokinetic parameters obtained from Chen et al. (2007).

Administration-approved model antiangiogenic agent) on the tumor penetration of topotecan after intraperitoneal and subcutaneous administration. The studies assessed the plasma and peritoneal pharmacokinetics of topotecan, tumor burden, tumor blood vessel density, liver topotecan exposure, and kidney topotecan exposure. For both studies, a human xenograft model for ovarian cancer was used, which was developed from 4- to 5-week-old, 20- to 25-g, male athymic nude mice.

In study A, peritoneal tumors were established in 30 animals by injecting ~2 million A2780 cells intraperitoneally. A2780 is an ovarian cancer cell line generated from an untreated patient. After tumor implantation, animals were randomly divided into two groups, treatment and control, with 15 animals in each group. Four days after tumor implantation, the treatment group started receiving anti-VEGF intraperitoneally at the dose of 5 mg/kg, whereas the control group received saline. Anti-VEGF and saline were administered twice weekly for 4 weeks. Four weeks after tumor implantation, both groups received intraperitoneal implants of ALZET micro-osmotic pumps (model 1003D), which delivered topotecan at the dose of 15 mg/kg over the period of 72 h. During infusion, at 24 and 48 h after pump implantation, blood samples were collected to assess the effect of anti-VEGF on the plasma pharmacokinetics of topotecan. At the end of the 72-h infusion, all animals were sacrificed to collect blood, intraperitoneal fluid, liver tissue, kidney tissue, and tumor samples. For each animal, visible tumor mass was collected and weighed to determine the total tumor burden. Tumors from each animal were divided into three aliquots for microvessel density determination, drug visualization via fluorescent microscopy, and for topotecan analysis via high-performance liquid chromatography (HPLC). Special care was taken to assure that the tumors of the same size, 0.5 to 1 cm in diameter, were used for comparisons between the treatment and control groups.

In study B, the effect of anti-VEGF on tumor penetration of subcutaneously delivered topotecan was examined. Tumors were established in 20 animals by injecting ~2 million A2780 cells intraperitoneally. After tumor implantation, animals were randomly divided into two groups, treatment and control, with 10 animals in each group. The dosing regimen of anti-VEGF was as described for study A. Four weeks after tumor implantation, both groups received implants of ALZET micro-osmotic pumps (model 1003D), placed in the subcutaneous space between the shoulders and along the back of the mice, which delivered topotecan at the dose of 15 mg/kg over the period of 72 h. During infusion, at 24 and 48 h after pump implantation, blood samples were collected to assess the effect of anti-VEGF on the plasma pharmacokinetics of topotecan. At the end of the 72-h infusion, all animals were sacrificed to collect blood, intraperitoneal fluid, liver tissue, kidney tissue, and tumor samples. For each animal, visible tumor mass was collected and weighed to determine the total tumor burden.

Concentrations of topotecan in plasma, tumor, and tissue samples were analyzed using the HPLC assay. The distribution of topotecan into tumor was assessed with the use of two-photon fluorescent microscopy. Immunohistochemistry for CD31 was performed on tumor samples to determine blood microvessel density. Data obtained from control and treatment groups were analyzed using the Student's *t* test (two sided, unpaired; *p* < 0.05 was considered significant).

HPLC Assay for Analysis of Topotecan Concentration. Topotecan concentrations in mouse plasma, intraperitoneal fluid, tumor samples, and tissue samples were analyzed using a validated reversed-phase high-performance liquid chromatographic assay (Chen and Balthasar, 2005). This assay measures topotecan concentration as the sum of lactone and carboxylate forms. Tumor and tissue samples were prepared by homogenization into a known volume of saline. The lower limit of quantitation for topotecan was 1 ng/ml in 20 μ l of mouse plasma, and recovery from tissue samples was more than 98%. Samples of peritoneal fluid were collected with preweighed filter paper, and the volume of collected fluid was determined by weight, assuming a density of 1 g/ml. Topotecan was extracted with methanol to allow assay via HPLC. Within- and between-day coefficients of variation were less than 13%, and the assay was linear over a concentration range of 1 to 2000 ng/ml.

Two-Photon Microscopy for Analysis of Topotecan Distribution within Tumors. Tumor samples were cut into 5- μ m cross-sections, obtained from the center of the tumor, and immediately mounted onto glass slides. To prevent drying, sections were hermetically sealed with the use of a coverslip, and then stored in a dark box at -80°C until analyzed, following the method of Errington et al. (2005). In all cases, samples were analyzed within 24 h from the time of collection. In brief, samples were subjected to two-photon excitation at 790 nm (Chameleon-Ultra Ti:Sapphire pulse laser; Coherent Inc., Santa Clara, CA). Emission was monitored between 460 and 630 nm. Fluorescent images were collected at the magnification of 20 $\times$ using a Leica TCS SP2 Spectral Confocal and Multiphoton Microscope (Leica Microsystems, Inc., Deerfield, IL). The repetition rate of 90 MHz was held fixed, and the pulse width was <140 fs at the peak.

Immunohistochemistry for Determination of Blood Microvessel Density. Tumor samples were fixed in formalin-free zinc fixative overnight. Samples were then dehydrated, paraffin-embedded, and sectioned to 5- μ m thickness. Each section was placed on a charged slide, dried in a 60°C oven for 1 h, and allowed to cool. Slides were dewaxed by three changes of xylene, followed by rehydration with 100, 95, and 70% ethyl alcohol and distilled water. Endogenous peroxidase was quenched with aqueous 3% H₂O₂. Slides were then processed in a Dako Autostainer (Dako North America, Inc., Carpinteria, CA). In brief, slides were washed with 0.05% Tween 20 in phosphate-buffered saline (PBS/T), followed by blocking with 0.03%

casein in PBS/T. Slides were then incubated with rat anti-mouse-CD31 monoclonal antibody (BD Pharmingen, San Diego, CA), or isotype-matched control antibody, at the concentration of 10 µg/ml. After a 0.05% PBS/T wash, slides were incubated with a biotinylated anti-rat secondary antibody (BD Pharmingen). Slides were then washed with 0.05% PBS/T and incubated with streptavidin peroxidase (Zymed Laboratories, South San Francisco, CA) and 3,3'-diaminobenzidine sequentially. Slides were counterstained with hematoxylin, washed, dehydrated with graded alcohol, and mounted with Permount (Thermo Fisher Scientific, Waltham, MA).

All CD31-positive intratumoral microvessels were counted at the magnification of 200×, following the method by Yin et al. (2006). Approximately 20 (range, 10–36) high-power microscopic fields (HPFs) per tumor were counted on each slide without selection (only necrotic areas were excluded). The average number of microvessels per HPF was determined for each tumor section. For each group, the results were presented as the average microvessel density per HPF.

Therapeutic Studies with Topotecan and Cisplatin. To establish a suitable peritoneal tumor model, approximately 2 million A2780 cells were injected intraperitoneally into athymic nude mice (20–25 g and 4–5 weeks old). Animals were randomly divided into 16 groups (TPT1–TPT8 and CDDP1–CDDP8). The number of animals assigned to each group and the treatment assigned to each group are summarized in Table 2. Topotecan was administered on a schedule of 5 mg/kg, delivered every 7 days for three cycles, as reported by Caserini et al. (1997). The dosing regimen for cisplatin, 4 mg/kg every 4 days for four cycles, was adapted from Sun et al. (1999).

For the groups TPT1 to TPT8, treatment started on day 6 after tumor implantation. Groups TPT6, TPT7, and TPT8 received anti-VEGF intraperitoneally at the dose of 5 mg/kg on days 6, 9, 13, 16, 19, and 23. Groups TPT4, TPT5, TPT7, and TPT8 received topotecan, 5 mg/kg i.p. or i.v. on days 8, 15, and 22. Control groups (TPT2, TPT3) received saline instead of anti-VEGF or topotecan. Likewise, groups CDDP6, CDDP7, and CDDP8 received anti-VEGF 5 mg/kg i.v. on days 6, 9, 13, 16, 19, and 23. Groups CDDP4, CDDP5, CDDP7, and CDDP8 received cisplatin, 4 mg/kg i.v. or i.p., on days 8, 12, 16, and 20. Control groups (CDDP2, CDDP3) received saline instead of anti-VEGF or cisplatin. As required by the University at Buffalo Institutional Animal Use and Care Committee, mice were removed from the study and euthanized when animal weight increased above 120% of baseline body weight (measured the day after tumor implantation). For purposes of assessment of the duration of animal survival, survival time was determined based on the date of animal death or removal from the study. Systemic

toxicity was assessed by observing changes into the animal body weight. Histopathological evaluation was performed by an experienced pathologist to examine animals for local and systemic drug toxicity. Percentage increase in lifespan, calculated as: $[(\text{median survival time in treated animals} / \text{median survival time in controls}) - 1] \times 100$, was used to compare therapeutic effectiveness of different groups. Kaplan-Meier survival plots and log-rank tests were used for statistical comparison between all the groups.

Histopathological Evaluation for Toxicity Assessment. To allow histopathological assessment, tissue samples were collected from the animals on the day of death or on the day of removal from the study. Based on the expected toxicity of the drugs, histopathological evaluation was performed on samples of sternum bone marrow, small intestine, kidney, liver, and spleen. In addition, any gross lesions in any organ seen at necropsy were also recorded. Three to five mice per group were studied. Samples were removed from moribund mice and fixed in 10% neutrally buffered formalin, processed conventionally for paraffin blocks. Paraffin sections (4 µm) were stained with hematoxylin and eosin. Toxicity was estimated based on the histo- and cytopathological changes and was graded semiquantitatively as weak, moderate, or severe compared with the normal organ histology by a board-certified pathologist (K.T.).

Statistical Analysis. Data are presented with 95% confidence intervals (CIs) in the text, and data in figures are presented as means with indication of S.D. values. Comparisons between groups were made by use of the Student's *t* test (two sided, unpaired); $p < 0.05$ was considered statistically significant. The Kaplan-Meier product limited method was used to calculate survival percentage. The log-rank test was used for statistical comparison of survival curves between all the groups using Prism software (GraphPad Software Inc., San Diego, CA). To account for the multiple comparisons made between different groups in the survival studies, the Bonferroni correction was applied. The *p* value of 0.05 was divided by the number of comparisons made for each study (i.e., 15), and statistical significance was concluded, where $p < 0.0033$. All statistical tests were two sided.

Results

Simulation Studies. Proof-of-concept investigations were initiated in silico using a hybrid, physiologically based pharmacokinetic model to predict the effect of changes in tumor blood flow on the tumor penetration of intraperitoneally or

TABLE 2
Summary of groups and treatments for the therapeutic studies with topotecan and cisplatin

Group	Treatment	Total Drug Dose	Total Anti-VEGF Dose	Animals per Group
		<i>mg/kg</i>		
TPT1	Control; no treatment	0	0	10
TPT2	Control; intravenous saline	0	0	10
TPT3	Control; intraperitoneal saline	0	0	10
TPT4	Topotecan; intravenously q7d × 3	15	0	10
TPT5	Topotecan; intraperitoneally q7d × 3	15	0	10
TPT6	Anti-VEGF; intraperitoneally 2×/week × 3	0	30	10
TPT7	Anti-VEGF; intraperitoneally 2×/week × 3 + topotecan; intravenously q7d × 3	15	30	10
TPT8	Anti-VEGF; intraperitoneally 2×/week × 3 + topotecan; intraperitoneally q7d × 3	15	30	11
CDDP1	Control; no treatment	0	0	10
CDDP2	Control; intravenous saline	0	0	10
CDDP3	Control; intraperitoneal saline	0	0	8
CDDP4	Cisplatin; intravenously q4d × 4	16	0	8
CDDP5	Cisplatin; intraperitoneally q4d × 4	16	0	8
CDDP6	Anti-VEGF; intravenously 2×/week × 3	0	30	8
CDDP7	Anti-VEGF; intravenously 2×/week × 3 + cisplatin; intravenously q4d × 4	16	30	8
CDDP8	Anti-VEGF; intravenously 2×/week × 3 + cisplatin; intraperitoneally q4d × 4	16	30	8

q7d, every 7 days; q4d, every 4 days.

intravenously infused topotecan. Results from the simulation studies are summarized in Fig. 3 and Tables 3 and 4. Consistent with previous experimental data and predictions made by the distributed model, the hybrid PBPK model showed an exponential decrease in topotecan concentration with increasing depth into the tumor (Fig. 3), after infusing the drug intraperitoneally. With simulated intraperitoneal infusion of topotecan, the model predicts that a 90% reduction in tumor blood flow will lead to a greater than 5-fold increase in the overall tumor topotecan concentration. Reducing blood flow by 50% was predicted to increase tumor concentrations approximately 1.5-fold (Table 3; Fig. 3). Given that antiangiogenic therapy has been found to reduce tumor blood vessel density by ~75% (Liang et al., 2006) and given the predictions of the model, we anticipated that anti-VEGF therapy would lead to a greater than 2-fold increase in tumor topotecan concentrations. It is noteworthy that this increased concentration is only predicted after regional administration of drug. In intravenous simulations (rather than intraperitoneal) administration of topotecan, reducing the tumor blood flow was predicted to lead to a modest decrease in topotecan concentrations in the tumor (Table 4). Irrespective of the route of drug administration, the reduction of tumor blood flow was not predicted to have an effect on the systemic and peritoneal pharmacokinetics of topotecan (Tables 3 and 4).

Pharmacokinetic Studies. To validate the results of simulations and to investigate the effect of antiangiogenic therapy on tumor drug exposure, two separate studies, studies A and B, were conducted. In study A, the effect of anti-VEGF on tumor penetration of intraperitoneally delivered topotecan was examined, whereas study B assessed the effect

Fig. 3. Concentration versus distance profile generated via simulations with the hybrid model. Topotecan was infused intraperitoneally at the dose of 15 mg/kg over the period of 72 h. To simulate the effect of antiangiogenic therapy on tumor blood perfusion, the normal tumor blood flow value of 0.06 ml/min/g was reduced by 50, 75, 90, and 99%. All simulations were performed with ADAPT-II software. Simulation results showed an exponential decrease in drug concentration with increasing depth into the tumor. Drug concentration in the tumor was predicted to increase as tumor blood flow was decreased. The model predicts that a 90% reduction in tumor blood will lead to a greater than 5-fold increase in the overall tumor topotecan concentration. ●, normal tumor blood flow (0.06 ml/min/g); ■, 50% reduction in normal blood flow; ▲, 75% reduction in normal blood flow; ○, 90% reduction in normal blood flow; □, 99% reduction in normal blood flow.

of anti-VEGF on the tumor penetration of topotecan after subcutaneous administration. In each study, drug concentrations were evaluated in plasma, peritoneal fluid, selected tissues (kidney, liver), and in tumor samples. At the time of collection of tissue samples and tumor samples (72 h after drug administration and more than 4 weeks after administration of tumor cells), there was considerable heterogeneity in the size and distribution of tumors in the peritoneum. In an effort to assure meaningful comparisons of tumor concentrations and microvessel density, assays were performed on tumors of similar size (0.5–1-cm diameter), obtained from sites adjacent to the mesentry.

After infusing topotecan intraperitoneally for 72 h, the group treated with anti-VEGF displayed a significant ($p = 0.0015$) increase in tumor topotecan concentration compared with the control group. The average tumor topotecan concentrations were 132 (95% CI, 95–168) and 834 (95% CI, 452–1216) ng/ml for the control and treatment groups, respectively. The observed 6.34 times increase in tumor topotecan concentration after treatment with anti-VEGF is similar to the 3- to 5-fold increase anticipated after simulations with the hybrid PBPK model.

Plasma concentration data for topotecan at 24, 48, and 72 h after the start of intraperitoneal infusion, obtained from study A, are summarized in Fig. 4. It was observed that the treatment with anti-VEGF did not have a significant effect on the systemic pharmacokinetics of topotecan. After 72 h of intraperitoneal infusion, apparent clearance values for the control and treatment groups were found to be 8.2 (95% CI, 6.8–9.6) and 9.5 (95% CI, 7.4–11.6) l/h/kg, respectively. At the end of intraperitoneal infusion, topotecan concentrations in peritoneal fluid were found to be approximately 135 times higher than concentrations of topotecan in plasma, which is similar to results reported in the literature (Andreopoulou et al., 2005). Anti-VEGF did not lead to a significant change in the peritoneal to plasma concentration ratio ($p = 0.81$). These results indicate that treatment with anti-VEGF does not have any significant effect on the systemic (Fig. 4) and peritoneal pharmacokinetics of topotecan. Treatment with anti-VEGF resulted in a significant ($p = 9.53 \times 10^{-10}$) reduction in the tumor burden of treated animals. Average tumor weight, described as percentage of body weight, for the control and treatment groups were 21% (95% CI, 18–24%) and 7% (95% CI, 6–8%). Treatment with anti-VEGF did not change exposure of topotecan in the liver or kidney ($p = 0.55$ and $p = 0.17$, respectively), two representative organs that reside within the peritoneal cavity.

Tumors collected from animals of study A were analyzed for drug distribution via two-photon fluorescent microscopy. Consistent with the results from HPLC analysis, two-photon fluorescent microscopy indicated, albeit qualitatively, that anti-VEGF treatment leads to a substantial increase in tumor concentrations of topotecan (Fig. 5A). Results from immunohistochemistry showed that there was a substantial reduction in tumor blood vessel density after treatment with anti-VEGF (Fig. 5B). Average microvessel densities for the control and treatment groups were found to be 67/HPF (95% CI, 62–73) and 24/HPF (95% CI, 23–26), respectively ($p = 2 \times 10^{-6}$).

Similar to the results shown from study A, data from study B also showed that the treatment with anti-VEGF did not lead to significant alterations in the systemic or peritoneal

TABLE 3

Results from the simulations^a

Simulation results depicting the effect of decreased blood flow on tumor topotecan concentrations after infusing the drug intraperitoneally.

Blood Flow to Tumor (ml/min/g)	0.006	0.015	0.03	0.06
Intraperitoneal concentration (ng/ml)	2697	2693	2691	2690
Blood concentration (ng/ml)	27	27.1	27.1	27.2
Concentration in tumor layer A (ng/ml)	574	293	171	102
Concentration in tumor layer B (ng/ml)	145	55.1	35.3	29.4
Concentration in tumor layer C (ng/ml)	54.7	30.3	27.6	27.2
Concentration in tumor layer D (ng/ml)	34.6	27.5	27.2	27.2
Concentration in tumor layer E (ng/ml)	30.1	27.1	27.1	27.2
Tumor homogenate concentration (ng/g)	334	166	100	65

^a All simulations were performed using the hybrid physiologically based pharmacokinetic model and were based on an animal weighing 25 g. Topotecan was infused either intraperitoneally or intravenously at the dose of 15 mg/kg over the period of 72 h. To simulate the effect of antiangiogenic therapy on tumor blood perfusion, different simulations were performed with varying values of tumor blood flow. For the purpose of simulation, the normal tumor blood flow value of 0.06 ml/min/g was reduced by 50, 75, and 90%. All simulations were performed with ADAPT-II software (University of Southern California).

TABLE 4

Results from the simulations^a

Simulation results comparing the effect of decreased blood flow on tumor topotecan concentrations after infusing the drug intraperitoneally or intravenously.

Blood Flow to Tumor (ml/min/g)	0.006	0.06
Intraperitoneal infusion of topotecan (15 mg/kg/72 h)		
Peritoneal concentration (ng/ml)	2697	2690
Plasma concentration (ng/ml)	27	27.2
Tumor homogenate concentration (ng/g)	334	65
Intravenous infusion of topotecan (15 mg/kg/72 h)		
Peritoneal concentration (ng/ml)	27.8	27.9
Plasma concentration (ng/ml)	92.6	92.6
Tumor homogenate concentration (ng/g)	85	92

^a All simulations were performed using the hybrid physiologically based pharmacokinetic model and were based on an animal weighing 25 g. Topotecan was infused either intraperitoneally or intravenously at the dose of 15 mg/kg over the period of 72 h. To simulate the effect of antiangiogenic therapy on tumor blood perfusion, different simulations were performed with varying values of tumor blood flow. For the purpose of simulation, the normal tumor blood flow value of 0.06 ml/min/g was reduced by 50, 75, and 90%. All simulations were performed with ADAPT-II software (University of Southern California).

pharmacokinetics of topotecan. Apparent clearance values for the control and treatment group were found to be 10.4 (95% CI, 8.6–12.3) and 13.4 (95% CI, 10.2–16.6) l/h/kg. Treatment with anti-VEGF did not alter the peritoneal to plasma concentration ratio ($p = 0.66$). Study B also showed that treatment with anti-VEGF resulted in a significant reduction in the tumor burden of animals ($p = 3.63 \times 10^{-4}$). Average tumor weights, described as percentage of body weight, for the control and treatment groups were 13% (95% CI, 10–16) and 5% (95% CI, 3–6), respectively. In mice treated with topotecan by subcutaneous infusion, anti-VEGF did not lead to a significant alteration in tumor topotecan concentrations ($p = 0.16$). Average tumor topotecan concentrations were 106 (95% CI, 52–160) and 63 (95% CI, 46–79) ng/ml for the control and treatment groups, respectively. Although not statistically significant, the trend toward a decrease in tumor topotecan concentrations in animals treated with anti-VEGF is consistent with predictions provided by the hybrid PBPK mathematical model. Treatment with anti-VEGF did not change exposure of topotecan in the liver or kidney ($p = 0.96$ and $p = 0.46$, respectively).

Therapeutic Study with Topotecan. Based on positive outcomes from the pharmacokinetic studies, we proceeded to evaluate the therapeutic effects of the proposed combination therapy. Survival curves for control and treatment groups are shown in Fig. 6A. Statistical comparison between the groups is described in Table 5. Three animals from the study were censored because two of them (one from TPT4 and one

Fig. 4. Effects of anti-VEGF on systemic pharmacokinetics of topotecan in tumor-bearing mice. After tumor implantation, animals were randomly divided treatment and control groups. Four days after tumor implantation, the treatment group started receiving anti-VEGF intraperitoneally at the dose of 5 mg/kg, whereas the control group received saline. Anti-VEGF and saline were administered twice weekly for 4 weeks. Four weeks after tumor implantation, both groups received intraperitoneal implants of ALZET micro-osmotic pumps (model 1003D), which delivered topotecan at the dose of 15 mg/kg over the period of 72 h. It was observed that the treatment with anti-VEGF did not have a significant effect on the systemic pharmacokinetics of topotecan; apparent clearance values for the control and treatment groups were found to be 8.2 (95% CI, 6.8–9.6) and 9.5 (95% CI, 7.4–11.6) l/h/kg, respectively. Open bar, control group with no treatment; solid bar, treatment group that received 5 mg/kg anti-VEGF twice weekly.

from TPT7) died during administration of anesthesia, and one mouse from TPT5 died on day 10 because of an unknown reason; however, drug toxicity cannot be ruled out. The median survival times for control animals were 18 to 23 days. Survival curves for all treatment groups, except TPT4 (intravenous topotecan) and TPT5 (intraperitoneal topotecan), were significantly different from the data obtained from control groups ($p < 0.0033$). Treatment with intravenous and intraperitoneal topotecan resulted in 22 and 35% increases in lifespan, respectively. Treatment with anti-VEGF alone also resulted in 35% increase in lifespan. Combining anti-VEGF with intravenous topotecan increased the median survival time for tumor bearing mice to 37 days. However, the combination of anti-VEGF and intraperitoneal topotecan produced superior results, increasing lifespan by 117% (median survival time, 50 days). This combination was significantly better ($p < 0.0033$) than any other treatment group. Moreover, four of 11 animals from the anti-VEGF + intraperito-

Fig. 5. Effect of anti-VEGF treatment on tumor drug penetration and tumor blood vessel density. **A**, effect of anti-VEGF on tumor topotecan concentration, after intraperitoneal chemotherapy. The figure displays fluorescent images (20 $\times$) of samples obtained from the pharmacokinetic study (study A). Frozen tumor samples were cut into 5- μ m sections and analyzed by two-photon fluorescent microscopy, following the method of Errington et al. (2005). Comparison of the left and right panels demonstrates the effect of anti-VEGF on the tumor penetration of topotecan delivered by intraperitoneal infusion. Higher concentrations of topotecan (visualized with emission of green fluorescence) were present in tumors excised from animals treated with anti-VEGF compared with results obtained from the control group. Each picture represents a tumor section obtained from different animals. The white bar on each picture represents 150 μ m. **B**, effect of anti-VEGF treatment on tumor blood vessel density. Tumor samples were fixed in formalin-free zinc fixative overnight. Samples were then subject to the standard protocol for the immunohistochemical staining of CD31 to identify blood vessels. There was a substantial reduction in tumor blood vessel density after treatment with anti-VEGF. Average microvessel densities for the control and treatment groups were found to be 67/HPF (95% CI, 62–73) and 24/HPF (95% CI, 23–26), respectively ($p = 2E-06$). Comparison of the left and right panels demonstrates that anti-VEGF treatment resulted in decreased staining (colored brown in the figure) for CD31, consistent with the expected effect of anti-VEGF on blood vessel density (image at 100 $\times$). Each picture represents tumor sections obtained from different animals. The black bar on each picture represents 100 μ m.

Fig. 6. Results from the therapeutic studies. After tumor implantation, animals were randomly divided into 16 groups (TPT1–TPT8 and CDDP1–CDDP8). The number of animals assigned to each group and the treatment assigned to each group are summarized in Table 2. Groups TPT6, TPT7, and TPT8 received anti-VEGF intraperitoneally at the dose of 5 mg/kg on days 6, 9, 13, 16, 19, and 23. Groups TPT4, TPT5, TPT7, and TPT8 received topotecan, 5 mg/kg i.p. or i.v., on days 8, 15, and 22. Control groups (TPT2, TPT3) received saline instead of anti-VEGF or topotecan. Likewise, groups CDDP6, CDDP7, and CDDP8 received anti-VEGF 5 mg/kg i.v. on days 6, 9, 13, 16, 19, and 23. Groups CDDP4, CDDP5, CDDP7, and CDDP8 received cisplatin, 4 mg/kg i.v. or i.p., on days 8, 12, 16, and 20. Control groups (CDDP2, CDDP3) received saline instead of anti-VEGF or cisplatin. Survival plots are displayed in the figure. **A**, survival fractions from therapeutic studies with topotecan. The combination of anti-VEGF and intraperitoneal topotecan led to superior results, relative to all other treatments. ●, TPT1, control, no treatment; ▼, TPT2, control, saline intravenously; ■, TPT3, control, saline intraperitoneally; ▲, TPT4, topotecan, 15 mg/kg i.v.; ○, TPT5, topotecan, 15 mg/kg i.p.; ◇, TPT6, anti-VEGF, 30 mg/kg; □, TPT7, topotecan, 15 mg/kg i.v. + anti-VEGF, 30 mg/kg; △, TPT8, topotecan, 15 mg/kg i.p. + anti-VEGF, 30 mg/kg. **B**, survival fractions for therapeutic studies with cisplatin. Consistent with expectations and with the topotecan study, the combination of anti-VEGF and intraperitoneal cisplatin led to superior results, relative to all other treatments. ●, CDDP1, control, no treatment; ▼, CDDP2, control, saline intravenously; ■, CDDP3, control, saline intraperitoneally; ▲, CDDP4, cisplatin, 16 mg/kg i.v.; ○, CDDP5, cisplatin, 16 mg/kg i.p.; ◇, CDDP6, anti-VEGF, 30 mg/kg; □, CDDP7, cisplatin, 16 mg/kg i.v. + anti-VEGF, 30 mg/kg; △, CDDP8, cisplatin, 16 mg/kg i.p. + anti-VEGF, 30 mg/kg.

neal topotecan combination group were cured, and they were defined as free of grossly detectable tumors at the end of the experiment (65 days). There was no substantial weight loss in any of the treatment groups, suggesting an absence of significant systemic toxicity in all groups. Toxicological as-

TABLE 5

Statistical analysis of the results obtained from therapeutic studies: study A

Percentage increase in lifespan, calculated as: [(median survival time in treated animals/median survival time in controls) - 1] × 100. Log-rank tests were used for statistical comparison between all the groups.

Group	Treatment	MDD (Range)	Change in Lifespan	Survivors Group	P Value (Control)	P Value (TPT4)	P Value (TPT5)	P Value (TPT6)	P Value (TPT7)
			%						
TPT1	Control; no treatment	23 (12–31)		0/10	1				
TPT2	Control; intravenous saline	18 (12–31)		0/10					
TPT3	Control; intraperitoneal saline	23 (6–35)		0/10					
TPT4	Topotecan; intravenously q7d × 3	28 (16–40)	22	0/10	0.088	1			
TPT5	Topotecan; intraperitoneally q7d × 3	31 (12–45)	35	0/10	0.015	0.25	1		
TPT6	Anti-VEGF; intraperitoneally 2×/week × 3	31 (20–45)	35	0/10	0.0029	0.125	0.841	1	
TPT7	Anti-VEGF; intraperitoneally 2×/week × 3 + topotecan; intravenously q7d × 3	37 (35–50)	61	0/10	<0.001	0.0065	0.145	0.141	1
TPT8	Anti-VEGF; intraperitoneally 2×/week × 3 + topotecan; intraperitoneally q7d × 3	50 (40–60+)	117	4/11	<0.001	<0.001	<0.001	<0.001	0.0015

MDD, median day of death.

assessment of bone marrow, small intestine, liver, kidney, and spleen showed that neither topotecan nor anti-VEGF, alone or in combination, induced any cytological or histopathological lesions in the animals examined.

Therapeutic Study with Cisplatin. To examine whether the effect of anti-VEGF was specific to topotecan or potentially generalizable to other chemotherapeutics, we conducted additional studies using a first-line chemotherapeutic agent for ovarian cancer, cisplatin. Survival curves for control and treatment groups are shown in Fig. 6B. Statistical comparisons between the groups are described in Table 6. All survival curves for treatment groups were significantly different from the control groups ($p < 0.0033$), which were associated with median survival times of 26 to 27 days. Treatment with intravenous and intraperitoneal cisplatin provided 15 and 35% increases in lifespan, and treatment with anti-VEGF alone resulted led to a 44% increase in lifespan. Combined anti-VEGF with intravenous cisplatin produced a 69% increase in lifespan, and the median survival

time for this treatment combination was 44 days. However, consistent with the topotecan data, and consistent with our expectations, the combination of anti-VEGF and intraperitoneal cisplatin produced superior results. This combination resulted in a 112% increase in lifespan, which was significantly better than results found from groups receiving intravenous cisplatin, intravenous cisplatin + anti-VEGF, and anti-VEGF alone. Among all groups, the maximal mean nadir weight loss was 6%, suggesting an absence of any severe systemic toxicity. No histopathological lesions were seen in the control (CDDP1–CDDP3) or intravenous cisplatin (CDDP4)- or anti-VEGF alone (CDDP6)-treated groups. In the cisplatin intraperitoneal group, one of four animals had mild renal and intestinal toxicity, and there was mild to moderate liver toxicity in the anti-VEGF + cisplatin combination treatment groups. Overall, the observed toxicity in all treatment groups was considered to be weak to moderate and, in the opinion of the pathologist (K.T.), would not influence animal survival.

TABLE 6

Statistical analysis of the results obtained from therapeutic studies: study B

Percentage increase in lifespan, calculated as: [(median survival time in treated animals/median survival time in controls) - 1] × 100. Log-rank tests were used for statistical comparison between all the groups.

Group	Treatment	MDD (Range)	Change in Lifespan	Survivors Group	P Value (Control)	P Value (CDDP4)	P Value (CDDP5)	P Value (CDDP6)	P Value (CDDP7)
			%						
CDDP1	Control; no treatment	26 (26–29)		0/10	1				
CDDP2	Control; intravenous saline	26 (26–33)		0/10					
CDDP3	Control; intraperitoneal saline	27(26–29)		0/8					
CDDP4	Cisplatin; intravenously q4d × 4	30 (29–36)	15	0/8	<0.001	1			
CDDP5	Cisplatin; intraperitoneally q4d × 4	35 (29–61+)	35	1/8	<0.001	0.027	1		
CDDP6	Anti-VEGF; intravenously 2×/week × 3	37 (34–40)	42	0/8	<0.001	<0.001	0.873	1	
CDDP7	Anti-VEGF; intravenously 2×/week × 3 + cisplatin; intravenously q4d × 4	44 (40–47)	69	0/8	<0.001	<0.001	0.0802	<0.001	1
CDDP8	Anti-VEGF; intravenously 2×/week × 3 + cisplatin; intraperitoneally q4d × 4	55 (44–61)	112	0/8	<0.001	<0.001	0.131	<0.001	<0.001

MDD, median day of death.

Discussion

In the context of regional drug administration, pharmacokinetic theory suggests that a reduction in tissue blood flow will reduce the rate of drug removal from tissue, allowing greater drug penetration into tissue (Dedrick and Flessner, 1997; Waniewski et al., 1999). There are few reports that have investigated drug penetration into tissue after alterations in blood perfusion. Ward and Jain (1988) observed that high doses of glucose can produce large decreases in tumor blood flow. Scheim and Lin (1993) subsequently hypothesized that glucose may be used as an adjunct to intraperitoneal chemotherapy and observed that the use of glucose containing peritoneal dialysate solutions increased drug penetration into peritoneal tumors, presumably as a result of decreased tumor blood perfusion. However, the high concentrations of glucose in dialysate solutions led to severe hyperglycemia, and it has been concluded that this adjunct therapy is not viable for clinical use. Duvillard et al. (1999) investigated the effect of epinephrine on the penetration of cisplatin and patent blue dye into abdominal tumors after intraperitoneal dosing. They observed that epinephrine strongly enhanced the penetration of the dye into tumor nodules. Moreover, combining epinephrine with intraperitoneal cisplatin also dramatically improved the antitumor efficacy of cisplatin. However, epinephrine effects on blood perfusion were not limited to the peritoneal tumor, and high-dose intraperitoneal epinephrine has been found to lead to unacceptable systemic and local toxicity.

We have proposed that antiangiogenic therapy may allow tumor-specific changes in blood perfusion and drug penetration, leading to increased efficacy of intraperitoneal chemotherapy of peritoneal tumors. It was reported that antiangiogenic therapy decreases tumor vessel diameter, density, and permeability (Jain, 2001). In addition, antiangiogenic agents can significantly reduce vascular volume and the vascular surface area in treated tumors (Hu et al., 2002). Antiangiogenic agents are known to affect neovascularization, making their effects specific for the tumor (Gerber and Ferrara, 2005). Based on these observations and the known determinants of capillary washout of drug after intraperitoneal chemotherapy, we hypothesized that antiangiogenic therapy will allow a tumor-selective increase in drug exposure, allowing for improved efficacy of intraperitoneal chemotherapy, without increasing local (peritoneal) exposure or toxicity and without increasing systemic exposure or toxicity.

Proof-of-concept investigations for the proposed hypothesis were initiated *in silico* as pharmacokinetic modeling is an inexpensive and insightful way to examine the viability of pharmacokinetic hypotheses. Here, we have developed a hybrid physiologically based pharmacokinetic model to predict the effect of changes in tumor blood flow on the tumor penetration of intraperitoneally or intravenously infused topotecan (a model chemotherapeutic agent). Our main objectives were to determine: 1) the effect of antiangiogenic therapy on systemic and peritoneal pharmacokinetics of topotecan, 2) the magnitude of change in tumor topotecan concentrations that may be achievable with combined intraperitoneal chemotherapy and antiangiogenic therapy, and 3) differences between combinations of antiangiogenic therapy and intraperitoneal or intravenous chemotherapy. Simulations showed that tumor-specific reduction in blood flow, as achiev-

able by antiangiogenic therapy, did not change plasma or peritoneal fluid concentrations of topotecan after either intraperitoneal or intravenous chemotherapy. This implies that combining antiangiogenic therapy with intraperitoneal chemotherapy will not change systemic or peritoneal pharmacokinetics of the chemotherapeutic agent and, hence, will not affect the high peritoneal/plasma concentration ratio achieved after intraperitoneal chemotherapy. The simulations also predicted that antiangiogenic therapy would increase tumor concentrations of topotecan substantially after intraperitoneal chemotherapy but lead to a slight reduction in tumor concentrations after intravenous chemotherapy.

Based on the favorable results from the simulation studies, we proceeded to test the effects of antiangiogenic therapy in a mouse model of human ovarian cancer. The model was developed by injecting A2780 cells, in suspension, intraperitoneally in nude mice to represent an optimally debulked tumor with residual microscopic disease. As predicted by the simulations, pharmacokinetic studies showed that drug concentrations in the systemic circulation and in peritoneal fluid were insensitive to antiangiogenic therapy, irrespective of the route of drug administration. Moreover, pharmacokinetic studies also showed that combining anti-VEGF with systemic or intraperitoneal chemotherapy did not change the exposure of the drug to liver or kidney, two representative organs that reside within the peritoneal cavity. These results were anticipated because the effects of antiangiogenic agents are limited to developing blood vessels; hence, anti-VEGF was not expected to affect blood perfusion or drug exposure in organs or tissues with mature blood vessels. The pharmacokinetic studies also showed that the combination of anti-VEGF and intraperitoneal topotecan increased tumor topotecan concentrations ~6.5-fold (relative to results observed in animals receiving intraperitoneal topotecan alone). However, anti-VEGF did not lead to increased tumor topotecan concentrations after systemic (subcutaneous) administration of topotecan.

The interesting observations (and predictions) of a beneficial effect of antiangiogenic therapy on tumor concentrations after intraperitoneal, but not intravenous, chemotherapy may be explained by considering the mechanisms associated with drug uptake and loss from tumor. The concentration of drug in the tumor, at steady state, represents a balance between the steady-state rate of drug entry into the tumor (K_{in}) and the clearance (CL) of drug from the tumor (K_{in}/CL). In the case of peritoneal tumors after intraperitoneal chemotherapy, the drug entry rate may be expected to be approximated Fick's Law of Diffusion, where K_{in} is a function of the permeability – surface area product and the steady-state concentration gradient between drug in peritoneal fluid and tumor ($C_{peritoneal} - C_{tumor}$). The CL of drug from the peritoneal tumor may be expected to follow Fick's Law of Perfusion, where CL is approximated by tumor blood flow (Q). Reductions in tumor blood flow, as achievable with antiangiogenic therapy, are expected to reduce CL with no effect on K_{in} . As such, antiangiogenic therapy may be expected to increase the concentration of drug in the tumor after intraperitoneal chemotherapy. On the other hand, after systemic chemotherapy, the rate of drug entry into the tumor and the rate of drug loss from the tumor are both driven by perfusion (i.e., flux = $Q \times [C_{blood} - C_{tumor}]$, $K_{in} = Q \times C_{blood}$, and $CL = Q$). With a reduction in blood flow to the tumor, there is a parallel

decrease in K_{in} and CL; thus, antiangiogenic therapy provides no improvement in tumor exposure after systemic chemotherapy.

It is important to note the simple mathematical model presented in this work assumes uniform blood flow and does not take into account complexities such as the existence of necrotic regions, heterogenous vascularization, high interstitial pressure, and potential differences in the nature of the extracellular matrix and effective drug diffusivity. The model has been designed for use in evaluating the effect of reductions in tumor blood flow, as potentially achieved by antiangiogenic therapy, on drug distribution into well vascularized regions of peritoneal tumors. The simulations evaluated changes in tumor blood flow in isolation from other possible effects achieved by antiangiogenic therapy. It is well known that antiangiogenic therapy influences capillary permeability and the structure of capillary beds, "normalizing" the tumor vasculature (Jain, 2001). Because of this normalization process, there may be an improvement in the functional efficiency of remodeled tumor vessels, which may enhance the delivery and antitumor activity of systemic chemotherapy (i.e., an effect opposite from the predictions of the mathematical model presented in this work). The "normalization" hypothesis by Jain was supported by a recent study from Dickson et al. (2007), which reported that, with systemic chemotherapy, drug penetration into neuroblastoma xenografts was enhanced after 3 days of anti-VEGF treatment. However, effects of anti-VEGF on the tumor vasculature are expected to be time-dependent, where long-term therapy is expected to lead not to normalization but to the destruction of tumor vessels. The Dickson et al. (2007) study reported that the beneficial effects of anti-VEGF on drug delivery to tumors were abolished after 7 days of anti-VEGF therapy.

During the collection of tumor samples in the pharmacokinetic studies, we observed that anti-VEGF treatment resulted in a significant reduction in the tumor burden of treated animals. It has been reported (Fujita et al., 2007) and observed by us (unpublished data) that anti-VEGF does not induce direct cytotoxic effects on tumor cells; consequently, the observed reduction in tumor burden is most probably resultant from indirect effects of anti-VEGF on the growth of tumor cells, presumably achieved by decreasing the availability of oxygen and nutrition.

The pharmacokinetic determinants of capillary removal of drug from peritoneal tumors (i.e., tumor blood flow, capillary surface area, and capillary permeability) are relevant for all chemotherapeutic drugs; hence, we hypothesize that antiangiogenic therapy will increase tumor exposure and antitumor efficacy for administration of any chemotherapeutic drug after regional administration (e.g., intraperitoneal chemotherapy of peritoneal tumors). To examine whether the pharmacokinetic effect benefits of anti-VEGF therapy translate to therapeutic benefit and to examine whether the effects of anti-VEGF were specific to topotecan or potentially generalizable to other chemotherapeutics, we conducted therapeutic studies with topotecan and an additional drug, cisplatin. Of all the groups tested, it was observed that the group with the proposed combination therapy (anti-VEGF + intraperitoneal topotecan, anti-VEGF + intraperitoneal cisplatin) provided the superior therapeutic results, without any increase in severe local or systemic toxicity. Combinations of antiangio-

genic therapy and intraperitoneal chemotherapy resulted in 117 and 112% increases in lifespan for topotecan and cisplatin, respectively. Moreover, the combined anti-VEGF and intraperitoneal topotecan therapy also resulted in a complete cure of the 36% of animals.

The clinical application of intraperitoneal chemotherapy typically involves administration of a large volume of fluid into the peritoneum (e.g., 2–4 liters), with the intent of maximizing drug exposure on all surfaces within the cavity. The intent is to reduce the potential for "sanctuaries" within the peritoneum, where tumor cells receive little or no drug exposure. In the present study, for reasons of technical feasibility, drug was infused in small fluid volumes. It is possible that this dosing protocol leads to suboptimal exposure in the peritoneum; as a consequence, it is possible that the present data provide a conservative measure of efficacy in this A2780 peritoneal tumor model.

It is noteworthy that we expect that the beneficial effect of antiangiogenic therapy on drug concentrations in tumors will be realized only after regional administration of drug and only in situations where antiangiogenic therapy may be applied to decrease tumor blood flow. We do not expect that the proposed therapy will provide substantial benefit in the treatment of mature human tumors with established blood vessels. The combination therapy may be applied most effectively to treatment of ovarian cancer patients after optimal debulking surgery, where tumor load is reduced to the level of "minimal residual disease." Early administration of antiangiogenic therapy to such patients may prevent rapid vascularization and growth of the residual peritoneal tumors, allowing for improved drug exposure after intraperitoneal chemotherapy.

In summary, we have used pharmacokinetic theory to develop the hypothesis that antiangiogenic therapy would lead to increased drug exposure in peritoneal tumors after intraperitoneal chemotherapy. This hypothesis was evaluated *in silico* with the use of a physiologically based pharmacokinetic model, and simulation results predicted a substantial increase in the tumor exposure to topotecan, which was used as a model anticancer drug. *In vivo* pharmacokinetic studies supported the hypothesis and demonstrated that administration of an anti-VEGF monoclonal antibody led to no changes in drug disposition in peritoneal fluid, in plasma, or in tissues with mature vasculature (e.g., liver and kidney) but did lead to a greater than 6-fold increase in topotecan concentrations in peritoneal tumors. Consistent with predictions of the model, this effect was observed after intraperitoneal administration of topotecan but not after systemic administration of the drug. Combined administration of anti-VEGF and intraperitoneal chemotherapy was found to produce superior anticancer effects in a mouse model of human ovarian cancer, using two chemotherapeutic agents (topotecan and cisplatin) in two separate studies. This study may serve as an example for the use of pharmacokinetic simulations to evaluate novel drug combinations, and the study results may be viewed as strong justification for the clinical evaluation of combined antiangiogenic therapy and intraperitoneal chemotherapy for treatment of peritoneal tumors, including those found in ovarian cancer.

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